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Left–Right Asymmetry: Cilia and Calcium Revisited

Leftward flow generated by motile cilia is known to underlie left–right asymmetry in vertebrate embryos. A new study now links intraciliary calcium oscillations to cilia motility and the downstream nodal signaling cascade that drives left-sided development.

Martin Blum* and Philipp Vick

When Johann Friedrich Meckel the Elder (1724–1774) prepared the visceral organs of a deceased 40-year old man (Figure 1), he probably was unaware of how lucky he was. His grandson, Johann Friedrich Meckel the Younger (1781–1833), to whom we owe Meckel's cartilage and diverticulum and the description of Meckel (Gruber) syndrome, used this specimen in his doctoral thesis *De cordis conditionibus abnormibus* ('about irregular conditions of the heart'). He referred to the unique features of this torso as "a total inversion of all organs of the chest and abdomen". Meckel the Elder was lucky because this condition is quite rare: *situs inversus totalis* only occurs in one out of ten thousand humans [1]. In 1933, the Swiss doctor Manes Kartagener described a human syndrome, in which situs inversion was much more abundant and affected about fifty percent of patients, which in addition suffered from impaired mucus clearance from the airways [2]. Finally, in 1976, Afzelius linked cilia to organ situs by showing that Kartagener individuals have immotile cilia [3]. Present day textbook knowledge has it that the left-sided Nodal signaling cascade drives asymmetric organ morphogenesis and placement in the vertebrate embryo, and that motile cilia initiate this event by generating a flow of extracellular fluids from right to left a little earlier [4]. How this fluid flow triggers the Nodal signaling cascade has remained a matter of intense research and debate ever since Hirokawa and colleagues described leftward flow for the first time in 1998 [5]. Calcium ions have been found asymmetrically in the cytoplasm [6] as a result of leftward flow, but how cilia, calcium and the Nodal cascade are connected has remained enigmatic. A new joint study by the Brueckner and Sun labs [7], published in this issue of *Current Biology*, now shows that cytoplasmic calcium waves are preceded by intra-ciliary calcium oscillations, which in turn depend on ciliary motility.

Brueckner and colleagues [7] have gone a long way from their discovery of two cilia types at the mouse left–right organizer (LRO), which has been the basis for the two-cilia model of flow-dependent symmetry breakage [6,8]. In this view, motile cilia at the center of the LRO produce an asymmetric left-directed flow of extracellular fluids (Figure 2A,B). This

Figure 1. Human torso with complete inversion of asymmetric organ placement (*situs inversus totalis*).

Historical preparation by Meckel the Elder. Please note that the apex of the heart (h) points to the right, the stomach (s) is placed on the right side while liver (l) and caecum (c) are found on the left. Photograph by Janos Stekovics [20], artwork by Bernd Schmid.

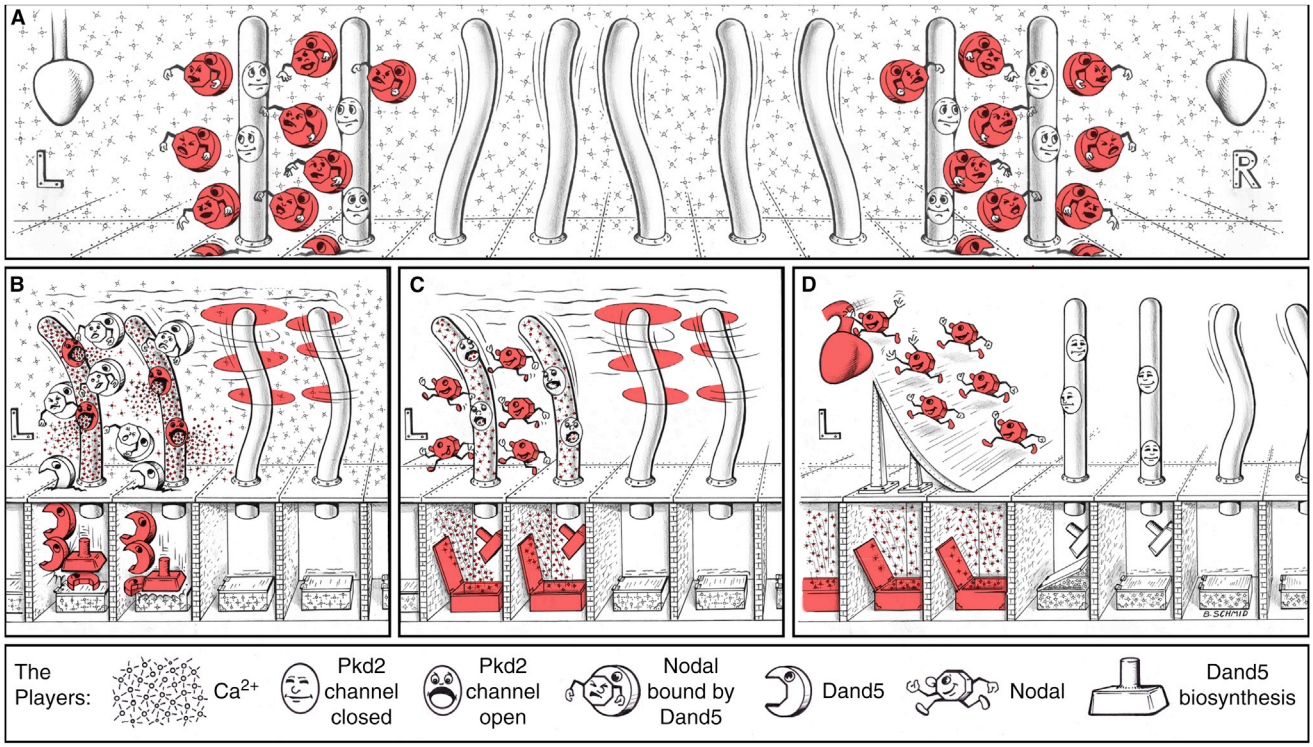

Figure 2. Cilia-driven leftward fluid flow breaks symmetry — the story and beyond.

(A) Left-right organizer (LRO) before flow: Dand5 keeps Nodal at bay. (B) Left side of LRO: flow bends sensory cilia at the left side of the LRO, enabling calcium entry into the cilium through the calcium channel Pkd2. (C) Left side of LRO: cytoplasmic calcium release from the endoplasmic reticulum inactivates Dand5 biosynthesis. (D) Calcium spreads to neighboring cells, paving the way for Nodal to reach the heart anlage on the left. Crate: endoplasmic reticulum. Artwork by Bernd Schmid.

leftward flow is thought to bend immotile sensory cilia at the periphery of the LRO to initiate the Nodal cascade at a distance (in the left lateral plate mesoderm) and in turn to induce asymmetric organ development and placement (Figure 2). Elegant work by Hamada's lab in Japan has provided proof of the sensory function of cilia in Nodal cascade induction [9]. Artificially applied fluid flow was able to rescue the cascade in a mouse model of Kartagener syndrome, i.e. in embryos without flow due to immotile cilia [9]. This rescue was dependent on the calcium channel Pkd2, short for polycystic kidney disease, a syndrome that develops in humans when *Pkd2* is mutated [10]. The dependence of cytoplasmic calcium asymmetry on Pkd2 was already noticed by Brueckner and colleagues in their ground-breaking report on the two types of cilia [6]. To link cilia to calcium asymmetry, Yuan *et al.* [7] generated dual reporters to detect and distinguish ciliary and cytoplasmic calcium oscillations. In renal cell cultures, a Pkd2 agonist induced ciliary calcium spikes, which preceded the increase of cytoplasmic calcium by 0.2–1.7 seconds. Renal cells are adequate cell types to test such reporters, as flow sensation in adult renal tubules and embryonic LROs share many similarities, including their dependence on Pkd2.

The true test of causality, however, was to analyze embryos, and here Yuan and colleagues used the zebrafish. The fish LRO, the spherical Kupffer's vesicle, differs in its overall morphology from that of the more or less flat epithelia of the mammalian node/posterior notochord or amphibian gastrocoel roof plate, but functionally all of these LROs are homologous [11], which is why the novel data impact on our understanding of flow-based symmetry breakage in vertebrates in general.

In zebrafish embryos, Yuan *et al.* [7] recorded highly dynamic intraciliary oscillations in response to leftward flow, predominantly on the left side of Kupffer's vesicle (Figure 2B). These oscillations were dependent on Pkd2 and on cilia motility, and ciliary oscillations were frequently followed by cytoplasmic calcium asymmetries in the same LRO and in neighboring cells, i.e. en route to the lateral plate mesoderm, the site of Nodal cascade induction (Figure 2C,D). Remarkably, asymmetric cytosolic calcium waves were found delayed in the embryo, as in renal cells: while ciliary calcium was most pronounced between the 1 and 4 somite stage, cytosolic calcium peaked when 5–9 somites had developed, i.e. several hours later [7]. Yuan *et al.* [7] went on to squelch calcium from the cilium by targeting the calcium-binding protein parvalbumin to LRO cilia. As a result, both intraciliary and cytosolic calcium spikes were lost in LRO and neighboring cells, concomitant with the later development of situs abnormalities, analyzed here as defects in heart looping [7].

Most interestingly, asymmetric expression of *Dand5* mRNA, the previously first molecular asymmetry functionally connected to leftward flow, was lost as well in these embryos [7]. *Dand5*, also known as *charon* in

zebrafish, *Cerl2* in mouse and *Coco* in *Xenopus*, encodes a secreted polyvalent growth factor inhibitor, which can bind to Nodal and inhibit its activity [12]. In the LRO, Dand5 is co-expressed with Nodal in the lateral cells perceiving leftward flow [13] (Figure 2A,B). As a consequence of flow, *Dand5* mRNA is down-regulated on the left side of LROs in fish, mouse and mammals, presumably lifting the repressive effect on Nodal to signal or transfer from the left side of the LRO to the left lateral plate mesoderm [14–16] (Figure 2C,D).

Unravelling the flow-dependent down-regulation of Dand5 is key to the understanding of symmetry breakage, as the induction of the Nodal cascade in the lateral plate mesoderm can be manipulated at will by side-specific manipulation of *Dand5*. In the *Xenopus* embryo, where side-specific manipulations can be performed, the cascade can be easily inverted [14]. All it takes is to inhibit ciliary motility, which prevents Dand5 down-regulation on the left, and at the same time to knock-down Dand5 on the right side of the LRO [14]. The new study by Sun, Brueckner and co-workers [7] now demonstrates for the first time that flow- and Pkd2-dependent intraciliary calcium oscillations control Dand5 asymmetry at the top of the left–right signaling cascade, which culminates in asymmetric organ morphogenesis and placement. How calcium impacts on Dand5 is, therefore, the next challenge in understanding the molecular mechanisms of flow-driven symmetry breakage.

However, the picture is not just black and white as outlined above. Neither are calcium oscillations, both ciliary and cytoplasmic ones, restricted to the left side of Kupffer's vesicle [7], nor does flow down-regulate *Dand5* mRNA in one hundred percent of cases [14]. If Dand5 is regulated post-transcriptionally, as has been proposed, an initially ciliary calcium signal spreading to the cytoplasm would of course be ideally suited to let the cell know that it is time to get rid of Dand5.

There is one more thought that comes to mind when watching the movies by Yuan *et al.* [7] on calcium oscillations at the LRO, referring to the spreading of calcium beyond the LRO cells proper [7]. How about if this spread paves the way for Nodal to reach the lateral plate mesoderm (Figure 2D)? The pure release of repression through down-regulation of Dand5 does not necessarily suffice to direct Nodal efficiently to its target tissue. It has been demonstrated that connexins are required for the transfer of laterality cues from the LRO to the left lateral plate mesoderm [17,18]. The directed propagation of a calcium wave from cell to cell, directly through gap junctions or via connexin-mediated purinergic wave propagation as proposed [17], might result in an alteration of the extracellular matrix that enables or facilitates Nodal to move from cell to cell until it reaches the left side to induce the cascade [19]. In that sense, the study of Yuan *et al.* [7] is inspiring beyond linking cilia, calcium and flow.

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